EFFECT OF INOCULUMS CONCENTRATION, TEMPERATURE AND AGITATION RATE ON SOLVENT PRODUCTION FROM PALM OIL MILL EFFLUENT (POME) BY *CLOSTRIDIUM BEIJERINCKII* ATCC 51743

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I declare that this thesis entitled “Effect of Inoculums Concentration, Temperature and Agitation Rate on Solvent Production from Palm Oil Mill Effluent (POME) by *Clostridium Beijerinckii*” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

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ABSTRACT

Few decades ago, world is totally depends on fossil fuel as energy source that currently most economically available source for personnel and commercial used leads in diminishing interest on development fermentation of biofuel industry. Unfortunately, due to constant conflict in the oil-supply regions and depletion of fuels oils supply resulted in viable of solventogenic fermentation industry in the world. Besides that, combustion of fossil fuel will produce various particle and gas that contribute to crises of environmental problem such as acid rain as well as greenhouse effect which is key factor in global warming. Thus, pressing need in reviewing the solventogenic fermentation of biofuel from a viable alternative sustainable and renewable energy resources that more renewable, more efficient and safer for environment. Unfortunately, the solventogenic fermentation needs to overcome high cost of production substrate together with low fermentation yield in order to compete with solvent production from fossil fuel. Palm oil mill effluent (POME) is abundant agricultural waste in Malaysia, shows great potential in used directly as alternative fermentation substrate. The objective of this study is manly to study the effect of temperature, agitation rate and inoculums concentration as variable factors that significantly influenced solvent production from palm oil mill effluent (POME) by *Clostridium beijerinkii* ATCC 51743. Analysis of total solvent production was done by using gas chromatography and isobutanol has been used as internal standard. Results that obtain from the study effect of 5 to 25% inoculums concentration to total solvent concentration show that the total solvent concentration will decrease as inoculums concentration increase. The highest production of total solvent is at 10% of inoculums concentration with 0.719 g/L production. Besides that, the study effect of 25 to 35 °C temperature to the total solvent production shows that concentration of total solvent production will decreases as temperature increase. The highest production is 0.568g/L at 30°C. Meanwhile effect of 50 to 250rpm agitation rate to the total solvent production shows that as agitation increases, total solvent production will decrease where the highest total solvent production was recorded at 150rpm with 0.675g/L. It can be conclude that, the temperature, agitation rate and concentration inoculums will be the significant parameter that affected production solvent from POME by *Clostridium Beijerinckii* (ATCC 51743).In addition, applying the Response Surface Methodology (RSM) was recommended for this study in order to determine the optimum level in enhances the solvent production.

ABSTRAK

Beberapa dekad yang lalu, dunia bergantung sepenuhnya kepada bahan api fosil sebagai sumber tenaga kerana harga bahan api ketika itu yang murah menyebabkan penurunan minat terhadap perkembangan industry bahan api daripada proses fermentasi. Namun begitu, disebabkan konflik yang berterusan di negara pengeluar minyak dan pengurangan sumber minyak menyebabkan kemunculan semula industri fermentasi solventogenik menghasilkan bahan api di dunia. Selain itu, pembakaran bahan api menghasilkan pelbagai gas yang menyumbang kepada pencemaran alam seperti hujan acid dan kesan rumah hijau yang menjadi punca pemanasan global. Oleh itu, perlunya penekanan dalam mengembangkan proses fermentasi solventogenik menghasilkan bahan api daripada sumber alternative yang lebih baharu dan mesra alam. Tetapi fermentasi solventogenik menghadapi harga bahan mentah yang mahal dan juga kurangnya penghasilan produk akhir untuk bersaing dengan industry yang menghasilkan bahan api daripada bahan api fosil. Bahan buangan kelapa sawit atau dikenali sebagai (POME) ialah bahan buangan yang membebankan Malaysia dilihat mempunyai potensi untuk digunakan sebagai bahan fermentasi. Objective kajian ini adalah untuk mengkaji kesan kepekatan inoculums, suhu dan kadar pengoncangan terhadap penghasilan bahan pelarut daripada POME oleh bacteria *Clostridium Beijerinckii.* Produk yang dihasilkan akan di analisis menggunakan gas chromatography dilengkapi pengesan api ion (GC-FID) yang menggunakan isobutanol sebagai standard dalaman. Keputusan yang diperolehi melalui kajian 5-25% kepekatan inoculum terhadap penghasilan bahan pelarut dilihat bahawa penghasilan bahan pelarut semakin menurun dengan peningkatan kepekatan inoculum. Penghasilan bahan pelarut tertinggi ialah pada 10% kepekatan inoculums iaitu sebanyak 0.719 g/L. Selain itu, kajian suhu dari 25-35°C terhadap pengahasilan bahan larut mendapati pengahasilan bahan pelarut semakin menurun dengan peningkatan suhu. Penghasilan tertinggi direkodkan pada 30°C dengan pengasilan sebanyak 0.568 g/L. Sementara itu, kajian terhadap kadar pengoncangan 50-250rpm terhadap penghasilan

bahan larut mendapati penghasilan menurun dengan peningkatan kadar pengoncangan. Penghasilan tertinggi ialah pada 150 rpm dengan catatan 0.675 g/L. Ia boleh dirumuskan bahawa kepekatan inoculums, suhu dan kadar pengoncangan menunjukkan kesan signifikan kepada penghasilan bahan pelarut. Tambahan lagi, sebagai cadangan untuk meninggikan lagi penghasilan produk aplikasi dengan menggunkan cara tindakbalas permukaan atau RSM boleh digunakan dengan mendapatkan nilai pembolehubah yang optimum.

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NOMENCLATURE

ABE	Acetone – butanol – ethanol
BOD -	Biological oxygen demand
COD -	Chemical oxygen demand
°C -	degree Celcius
DNS -	Dinitrosalicylic acid
eg -	Example
GCFID -	Gas Chromatography(Flame Ionization Detector0
g -	gram
HPLC -	High Performance Liquid Chromatoghraphy
hr -	hour
L -	Liter
MT -	Metric tonne
ml -	mililiter
μm -	micrometer
min -	minute
POME -	Palm Oil Mill Effluents
UV – Vis	UltraViolet Vision

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Historically, late 1970s there was increases international interest to review production of alcohol fuels from easily and renewable resources (Qureshi *et al.,* 2000). The main factor increasing interest among various government and scientific communities to develop solvent fermentation process is due to increasing cost of petrochemical feedstock and high demand of co-solvent gasoline as energy source to generate energy in industrial sector. Brazil is the first country that introduced its own biofuels, ethanol production program followed by USA in 1977. In early 1980s, fermentation production biofuels could not compete with the economical creation of solvents from cheap, plentiful petroleum due to reduction in oils price lead to diminishing interest. However, constant conflicts in the oil-supply regions and depletion of fuels oils supply resulted to oil price increases make research activities on this fermentation is revival.

Nowadays, increases demand in chemical additive and liquid fuels create a pressing need to find a viable alternative sustainable energy. The solvent (acetone-butanol-ethanol) was define has many commercial application in various industries such as in oleochemical industries and also in manufacture of lacquers, resins and also increase demand in blend with gasoline as energy sources. Unfortunately, there was certain issues occurs that must facing in developing renewable energy resources that

more renewable, more efficient and safer for environment. For the first generation in solvent fermentation research is in bioethanol fermentation by yeast whereby production is derived from sugar, starches, and oils. These solvent are creating from crops that are competing with food crops for the use of agricultural land and water.

As result, solvent product from solvent fermentation becomes another attractive sustainable alternative due to its ability in production that derived from high carbohydrate waste of industries that abundant in certain country especially in Malaysia. Its inherent chemical properties make it superior than bioethanol for use in combustion engines. Biobutanol is product from anaerobic fermentation of carbohydrate to solvent by *Clostridium* species and has four carbons chains, make it has more energy than bioethanol that is for about 25% more energy per unit volume. It's also has lower vapor pressure and higher flashpoint than bioethanol making it easier to store and safer to handle. Contrast to properties of ethanol that attract to water, butanol is hydrophobic that can be blended at a refinery without requiring modifications in blending facilities, storage tanks or retail station pumps that can run in unmodified engines at any blend with petrol and may also blended with diesel and biodiesel. Furthermore, butanol is less corrosive than ethanol and can be transported using existing infrastructures.

The commercial manufacture of butanol via Acetone- Butanol- Ethanol (ABE) fermentation from non-food sources by using solventogenic clostridia has almost completely replaced petrochemical routes. Current research in ABE fermentation is directed at genetically altering *Clostridium spp.* to enhance final butanol yield. Ian (1989) state that by using *Clostridium acetobutylicum or C.beijerinckii* can help to minimize problem of all solvent fermentation process such as lower reactor productivities and problem in product recovery. In previous study reported by Ezeji *et al.*(2007), *C.beijerinckii* has great biotechnological potential for production of butanol, acetone and isopropanol due to abilities in utilize of broad substrate range (pentose, hexose, starch and others) besides can sustained production of solvents well into log-phase with respects to strain degeneration and adaptability in continuous processes.

There have several effects that identified contribute to the economic solvent price in industry that is high cost of substrate, low product concentration, and low solvent yields (Qureshi *et al.,* 2001). The most effected problem that made up to 60% for overall cost solvent production is cost of substrate. Thus, the availability of variety low cost feedstock as an inexpensive raw material including whey permeate, soy molasses, barley straw and other agriculture biomass culture make economically viable for ABE fermentation. Unfortunately, this substrate need to hydrolyzed first into simple sugar before use as medium in fermentation process (Qurehi *et al.,* 2010).

Besides that, inhibitor directly occurs after fermentation process contributes to lower product concentration makes widely research was done to discover the most reliable substrate to use. In Malaysia, Palm Mill Oil Effluent (POME) represents an alternative raw material for fermentation process that is attractive in both economic and geographical consideration. POME is one of agricultural waste that produces form production of cruel oil and the most polluted organic residues that abundant in Malaysia show great potential to be used directly as raw material for production of butanol without need to hydrolyzed first into simple sugar (Sahaid *et al.,* 2003). In addition, POME is industrial waste that containing 95-96% water, 0.6-0.7 % oil and 4-5% total solids that if untreated effluent is discharged into water sources will cause environmental problem (Wu *et al*., 2008). On the other hand, POME was identified to be a potential source to generate renewable bioenergy such as biofuel, biomethane and biohydrogen (Lam *et al.,* 2010).

Traditionally, technique of ‘one-factor at a time’ was applied widely in optimizing production for multivariable system. This preliminary study is seen very important in order to give the basic data before proceed with the optimization step for the solvent production by *C.beijerinckii.* Thus, estimation of several main effect and factor interaction in solvent production from various renewable resources becomes favorable in ABE fermentation process review.

1.2 Problem Statement

In Malaysia, development of renewable energy resources is become favorable due to decreasing availability fossil fuels source that now can only survive for another 20 to 30 years. An alternatives source for fuels and other petroleum based products is extremely needed due to global demand and unstable condition in worldwide petroleum prices. Furthermore, negative impact to environment generates by the worldwide utilization of fossil fuels besides problem that contribute from agricultural waste that most abundant in Malaysia get attention to study on. According to Claassen *et al*. (2000), the ABE fermentation has resurfaced due to new global support for the exploitation of biomass as a sustainable source of energy. Therefore, solvent production should be focus towards the use of renewable energy that more friendly to environment.

Unfortunately, solvent fermentation process by *C.beijerinckii* suffered from high cost of solvent production from high cost medium component. In previous study by Mariam *et al.* (2007), solvent production from food based crops was widely used by researcher such as corn, potato and sugar cane. But, an issue occurs that give negative impact on global food supply due to substrate used is competing human food based material resulted to high demand and fluctuation price in market. This becomes the main factor causing why ABE fermentation could not survive due to high cost of raw material for medium preparation. Therefore, studies in POME an agricultural residue that contain high concentrated organic compound become favorable as suitable with an economical substrate for solvent production.

Since 1950's, solvent fermentation process by clostridia is very complicated and difficult to control resulted to the limitations on production such as lower yields and lower productivity of product. David (2004) reported that, low solvent yield and solvent concentration due to severe product inhibition made solvent production from glucose by ABE fermentation is uneconomical. According to Christine *et al.* (2009), the main problem in ABE fermentation that needs to overcome is the low conversion of glucose to

solvent. Thus, study the effect of different parameters on solvent production need to be focus in order to increase production yield during fermentation process.

1.3 Objective

The aim of this study is want to study effect of inoculums concentration, temperature and agitation rate on solvent production by *Clostridium Beijerinckii* from Palm Oil Mill Effluent (POME).

1.4 Scope Of Study

To achieve objective of this study, there are several scope that have been identified:

i) To study the growth profile of *Clostridium Beijerinckii* in Palm Oil Mill Effluent (POME) and Reinforce Clostridium Medium (RCM).
ii) To study the effect of inoculums concentration (5 to 20%) on solvent production.
iii) To study the effect of temperature (30 to 50°C) on solvent production.
iv) To study the effect of agitation rate (40 to 160 rpm) on solvent production.
v) To study the glucose consumption at different inoculums concentration, temperature and agitation rate.

1.5 Rational & Significant Of Study

The uncertainty and depletion petroleum supply resulted in viable interest in traditional solvent (Acetone Butanol Ethanol) fermentation by *Clostridium* strain (Parekh *et al.,* 1998). Recently, world is facing problem in developing renewable energy that more renewable, more efficient and more safe in environment in order to switching

dependence on fossil fuels supply. Thus, widely study on solvent production was developed to help world in indicating another solvent from renewable resources.

For more than three decades, critics have tried to cast raw materials that used in production biofuels such as corn that said disturbing food chain in human life being. But nowadays, the dominant route for making biofuels was turn to material that renewable and economical substrate that viable source or abundant in each country that interest in ABE fermentation. In Malaysia, agricultural waste that abundant in peninsular of Malaysia that is palm oil mill effluent (POME) was proposed to use in fermentation process to produce solvent. POME is high contaminant agricultural residue that contain high mixture of carbohydrate such as starch, cellulose and other carbohydrate but can harmful to environment if not properly manage. It can be utilized by *C. beijerinckii* and able to produce high production of solvent compare to other substrate used such as barley straw, corn fiber and switch grass (Qureshi *et al.,* 2007). By using POME as raw material also can help to reduce price of solvent at international market that influence from cost of substrate.

Over the years, fermentation process by using anaerobic microorganisms provides a dominant review for converting biomass and agricultural waste into chemicals and biofuels. One of the largest acetone-butanol-ethanol fermentation (ABE fermentation) process ever developed in process industry is in 1917-1955 where applied widely bacterium that strictly anaerobic condition that is *Clostridium acetobutylicum*. Unfortunately, this strain is facing problem that produce lower yield in final product due to lack tolerance in acid production in final process. In order to overcome this problem and slightly increase solvent production, study in applying strain that tolerant to higher acid production such as *Clostridium beijerinckii* is need to focusing more (Tashiro *et al.,* 2010). In addition, further study is needed to screen the factor that effects the solvent production.

CHAPTER 2

LITERATURE REVIEW

2.1 Acetone-Butanol-Ethanol (ABE) Fermentation

In present, solvent that manufactured from industrial scale by ABE fermentation is not longer competing with chemical synthesis from petrochemical feedstock. However, due to high demand for solvent is industry besides development in biotechnology and bioprocessing field, interest in fermentation of solvent production as renewable energy has returned and current research was focusing directed towards the development of better processes and microbial hyper producing- strains. On the other hand, the strong reason in a revival of research activities on this field is the depressed crude oil world prices due to limited supply of fossil fuels and constant conflicts in oil-supply region (*Qureshi et al., 2007*).

Fermentation of ABE (acetone, butanol, and ethanol) fermentation involve a two-step that is acid production phase carried out by *clostridium* species and followed by solvent production acetone, butanol and ethanol in 3:6:1 wt ratio composition production (Qureshi *et al*.,2007). The uncertainty associate with petrochemical supplied resulted in interest to traditional fermentation by *clostridia*. In 1914, first industrial ABE fermentation was commercialized from starcy raw material by *Clostridium acetobutylicum* has discovered. Second generation was developed for butanol production due to development modern technology in biotechnology and bioprocess field. Unfortunately, production of butanol hampered with low of product yield due to butanol

itself toxicity to bacteria resulted to lower solvent yield and also faced to high cost of raw material. In response to overcome this problem, production of butanol from cheap renewable resource such as sugar crops (*Atsadawut et al., 2006*), lignocelluloses feedstock and agricultural residue (*Qureshi et al., 2010*) was develop.

Metabolic pathway that demonstrates among clostridia species such as *C.acetobutylicum, C.beijerinckii* and *C. saccharoperbutylacetonicum* is similar (Zheng *et al.,* 2009). Figure 2.1 shows metabolic pathways to ABE fermentation by *C. acetobutylicum.* Clostridia have a diverse group of anaerobic bacteria that can utilize variety of substrates including monosaccharides and polysaccharides (Sakuragi *et al.,* 2010). Glucose is supply through the glycolytic (EMP) pathway that generates pyruvate and 2 molecules of ATP and NADH. During fermentation, *C.acetobutylicum* will produce 3 major product classes that are solvent (acetone, ethanol and n-butanol), organic acid (acetic acid, lactic acid and butyric acid) and gases (carbon dioxide and hydrogen) (Sakuragi *et al.,* 2010). Metabolic reaction of ABE fermentation is represent in two phase that is acidogenic phase and solventogenic phase.

At acidogenic phase, accumulation of organic acid primarily acetate and butyrate will start. The synthesis of acetate and butyrate is important for generation of ATP that needed for cell growth and further metabolism synthesis. When the concentration of undissociated acid reaches some threshold value, second phase that is solventogenesis is triggered. During solventogenesis is the second phase of ABE fermentation, the acid that produce during acidogenic phase is reused for production of Acetone, Butanol and Ethanol. Pathway in production ethanol is started with reaction of acetyl-CoA and acetaldehyde dehydrogenase that will produce acetaldehyde, by then reacts with ethanol dehydrogenase will produce ethanol. On the other hand, production of acetone is interaction reaction between acetate and butyrate with involve phosphotrans acetylase that produce Acetyl P by then, with involving acetate kinase enzyme will produce acetone. The butanol production pathway from acetyl-CoA requires 5 enzymes that is that is thiolase (thl), 3-hyroxybutyryl-CoA dehydrogenase (hbd), 3-hydroxybutyryl-CoA

4.5 Solvent Production at Different Agitation Rate

Table 4.4: Solvent Production at Different Agitation Rate

Parameter		Solvent Production, g/L			Total Solvent Production (g/L)
		Acetone	Butanol	Ethanol	
Agitation Rate (rpm)	50	0.114	0.120	0.176	0.410
	100	0.126	0.170	0.248	0.544
	150	0.201	0.190	0.284	0.675
	200	0.180	0.140	0.250	0.570
	250	0.100	0.005	0.220	0.325

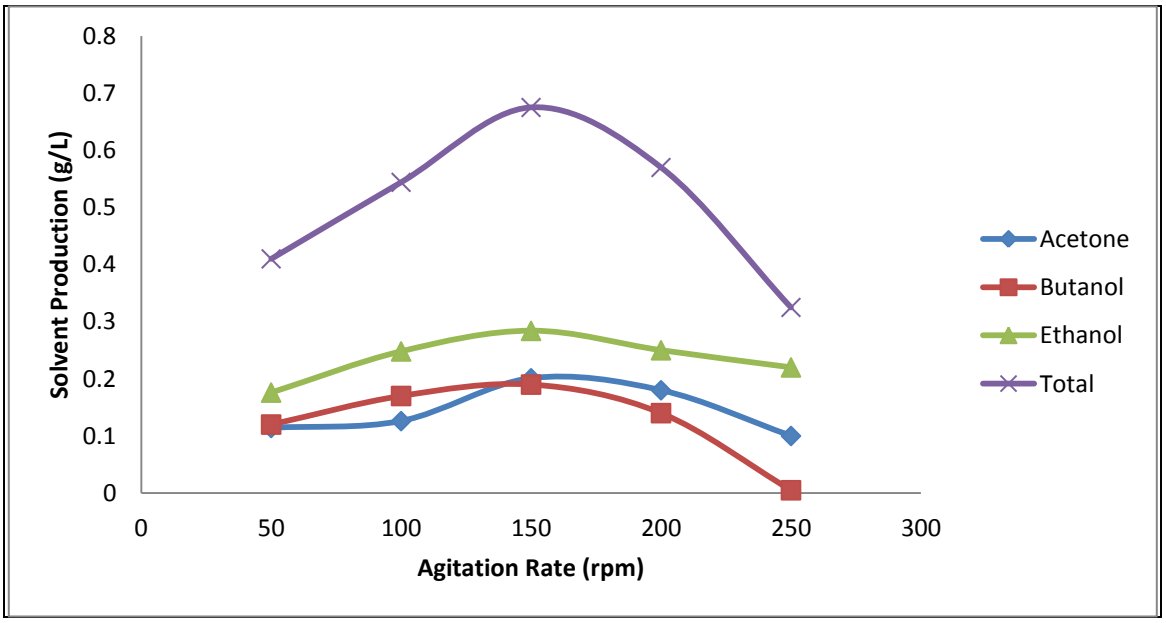

Figure 4.4: Solvent Production at Different Agitation Rate

Result in Table 4.4 showed that the solvent production was decreased significantly after 150 rpm. The presence of inactive or dead cell and also deficiency of nutrient were given earlier as possible reason for decreased total solvent production by *Clostridium Beijerinckii* during ABE fermentation (Ezeji *et al.*, 2003). Figure 4.4 showed that with the increases of agitation rate from 50 until 150 rpm will increase the